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Genetic confirmation of *Aricia artaxerxes* (Fabricius, 1793) (Lepidoptera, Lycaenidae) in the Czech Republic, its conservation significance and biogeographic context

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Abstract. We report here the first molecular evidence for the occurrence of *Aricia artaxerxes* (Fabricius, 1793) (Lepidoptera: Lycaenidae) in the Czech Republic. In Central Europe, this species may co-occur with its more common sibling, *Aricia agestis* (Denis & Schiffermüller, 1775). We sequenced the cytochrome c oxidase subunit 1 of darkly-coloured, putative *A. artaxerxes* specimens in the Czech Republic. We confirmed *A. artaxerxes* only from a limestone area in South Bohemia (Vyšenské kopce National Nature Reserve), which is probably the only locality of the species in the Czech Republic. This area is located at ca. 550 m A.S.L., showing that the elevation overlap with *A. agestis* could be high in Central Europe. Other surveyed individuals were confirmed as *A. agestis*, with a minimum p-distance of 1.98% between the two species. The South Bohemian area of occurrence is probably highly isolated (approx. 190 km) from localities of the species in neighbouring countries, highlighting the conservation importance of the *A. artaxerxes* population and of the insular calcareous areas in the Šumava Mountains foothills. We used database sequences of *A. artaxerxes* to place the Czech population into a wider phylogeographic context. The Czech population is monomorphic, consisting of a single haplotype, which is present from Scandinavia through Germany to Central Asia.

Introduction

Aricia artaxerxes (Fabricius, 1793) is a Palaearctic species of lycaenid butterfly, occurring in Europe together with several cryptic siblings: the widely distributed *Aricia agestis* (Denis & Schiffermüller, 1775), the West Mediterranean *A. cramera* (Eschscholtz, 1821) and *A. montensis* Verity, 1928. *Aricia agestis* and *A. artaxerxes*, which are sympatric in Central Europe, differ in larval and pupal morphology, and rearing was traditionally used to distinguish them (Kames 1976; Lepidopterologen-Arbeitsgruppe 1987; Warecki 2010). Several studies combining morphology and molecular markers (Aagaard et al. 2002; Dincă et al. 2011; Sañudo-Restrepo et al. 2013) concluded that these taxa indeed represent true species and can be distinguished by allozyme profiles or the cytochrome c oxidase subunit 1 (COI, i.e., the standard DNA barcode for animals), but not by wing pattern or genital morphology. More specifically, adults from Scotland (nominotypical *A. artaxerxes artaxerxes* Fabricius, 1793) usually bear a white discoidal dot on the upper side of each brown fore wing. However, adults from mainland Europe (*A. artaxerxes allous* (Hübner, 1819), and other areas, cf. Sañudo-Restrepo et al. 2013) lack that trait, differing from adult *A. agestis* only

by a darker overall colouration, due to smaller or obscured orange spots on the upper sides of the fore wings, but also smaller spots on the hind wings (Tolman and Lewington 2008, p. 130).

Ecologically and biogeographically, the majority of *A. artaxerxes* records appear concentrated in calcareous short-turf grasslands at high latitudes or elevations (Lepidopterologen-Arbeitsgruppe 1987; Asher et al. 2001; Aagaard et al. 2002; Sañudo-Restrepo et al. 2013; Pecsenye et al. 2014), where the species has a single annual generation from June to August. Its confirmed larval host plants across the range include *Helianthemum* Mill. spp. (Cistaceae; *H. nummularium* (L.) Mill. in Britain: Asher et al. 2001) and large-flowered *Geranium* L. spp. (Geraniaceae; *G. sanguineum* L. in Poland: Warecki 2010; see also Tuzov 2000; Gorbunov and Kosterin 2003). The distribution of *A. agestis*, in contrast, includes most of the European continent except for the highest latitudes, and extends to the Tian Shan in Asia. It has two or three annual generations in the lowlands of Central Europe, and three or even more in more southerly regions (Hesselbarth et al. 1995). Its host plant range overlaps with *A. artaxerxes*, as it includes both many Geraniaceae (e.g., *Geranium dissectum* L., *G. pusillum* Burm. fil., *G. pratense* L. and *G. molle* L., and *Erodium* L'Her. ex Aiton spp.) and *Helianthemum* spp. in natural conditions (Thomas et al. 2001; Buckley et al. 2012). Reflecting the broad ecological range of the host plants used, *A. agestis* inhabits a broad range of biotopes, from xeric grasslands to mesic and even alluvial meadows (Bury 2016). In European mid-latitudes, the single generation of *A. artaxerxes* coincides with the gap between spring and summer generations of *A. agestis* (Beneš et al. 2002). *Aricia artaxerxes* is usually associated with higher elevations than *A. agestis* in Central and Southern Europe, but their occurrence could overlap at intermediate elevations, causing problems with identification in the potential contact zones (cf. Dincă et al. 2011).

Until the late 20th century, the two species were not distinguished in the lowlands of Central Europe, including in the Czech Republic. The first records of *A. artaxerxes* for the country are from 1964 (Králíček et al. 1970) and 1971 (Králíček and Gottwald 1980), from the warm region of South-Eastern Moravia (foothills of the White Carpathians Mts). These and later records were mainly identified based on the combination of flight period and a darker colouration than the more common *A. agestis*. The distribution atlas of Czech butterflies (Beneš et al. 2002) considered only the records of *A. artaxerxes* in a single atlas grid square (Vyšenské Kopce National Nature Reserve [= NNR] near Český Krumlov, South Bohemia) as recent and relatively reliable. The Czech Republic Butterflies and Moths Recording Database (Institute of Entomology, Biology Centre of the Czech Academy of Sciences) together with the Information System of Nature Conservation (Nature Conservation Agency of the Czech Republic) register possible, unreliable records of *A. artaxerxes* from 38 grid squares (as opposed to 427 for *A. agestis* – 63% of the country´s area) (Fig. 1). The national Red list (Hejda et al. 2017) considers *A. artaxerxes* as critically endangered and *A. agestis* as of least concern.

The recent increase in the use of molecular markers for species identification, the emergence of national barcoding programmes (e.g., Dincă et al. 2011; Hausmann et al. 2011; Litman et al. 2018) and butterfly recording in European countries have significantly improved our knowledge of *A. artaxerxes* distribution. Besides the previously known distribution at high elevations, the species was reliably confirmed from relatively low elevations of Baden-Württemberg (at ca. 850 m A.S.L.), Saxony-Anhalt (ca. 430 m A.S.L.), and Thuringia (ca. 370 m A.S.L.) (Hausmann et al. 2011; and specimens used in Mutanen et al. 2016). It is also reported from central-northern Poland (Buszko and Maslowski 2008; Sielezniew and Dziekanska 2010), northern Hungary (800–850 m A.S.L.) (Pecsenye et al. 2014) and central-eastern Slovakia (600–800 m A.S.L.) (Reiprich and

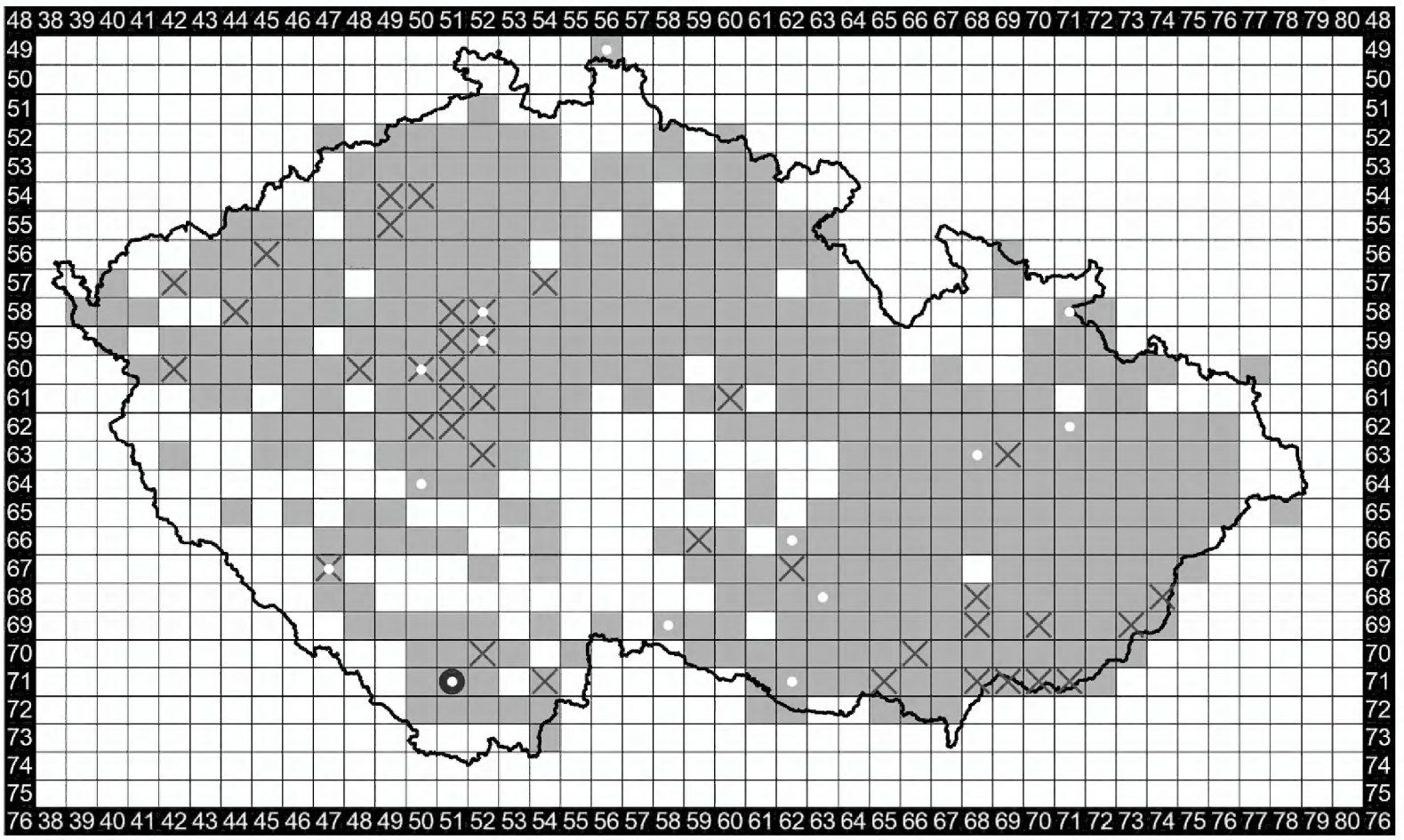

Figure 1. Distribution map of *Aricia artaxerxes* and *A. agestis* in the Czech Republic.The map was created based on 8464 records both historical and recent (until 2018) of these two species from The Czech Republic Butterflies and Moths Recording Database (Institute of Entomology, Biology Centre of the Czech Academy of Sciences) and from the Information System of Nature Conservation (Nature Conservation Agency of the Czech Republic). Grey squares: *A. agestis*, crosses: probably darkly-coloured *A. agestis* (putative *A. artaxerxes* records), white points: barcoded individuals, black circle: confirmed *A. artaxerxes*.

Okáli 1988–1989; Pecsenye et al. 2014; L. Víťaz, personal communication), but as far as we know, these populations have not been studied using molecular sequencing.

In this contribution, we use DNA barcodes to clarify the status of *A. artaxerxes* in the Czech Republic. Specifically, we barcoded material from the putative *A. artaxerxes* population near Český Krumlov, and individuals with missing or reduced orange spots on upper sides of the wings (hereinafter "dark") from several localities across the country with past *A. artaxerxes* reports, and a selection of typical *A. agestis* (with developed orange spots, hereinafter "light"). In addition to evaluating the current status of *A. artaxerxes* in the country, we place it into a wider biogeographic context and discuss its conservation significance.

Material and methods

We tested twelve individuals from the putative *Aricia artaxerxes* population from Vyšenské kopce NNR, South Bohemia (univoltine, dark, occurring between spring and summer broods of sympatric *A. agestis*; but also including reared individuals without reduced orange spots originating from dark females), five dark or intermediate individuals from other localities, and nine light individuals with developed orange spots (Table 1, Fig. 2). To place these individuals into a broader context, we further mined a total of 113 sequences from GenBank: *A. artaxerxes* (N=94), *A. agestis* (N=9), *A. anteros* (N=3), *A. cramera* (N=3) and *A. montensis* (N=4) (Suppl. materials 1, 3).

Table 1. Sampling sites of *Aricia artaxerxes* and *A. agestis* from the Czech Republic used for DNA-based identification.

Species	Voucher	Colouration	Locality	GenBank codes	Elevation [m A.S.L.]	Grid square
A. artaxerxes	6 specimens ZF-LY-001622 JB00138 ZF-LY-001774 to 1776, 1778	dark	Vyšenské kopce National Nature Reserve, South Bohemia	MN107398 – MN107403	550	7151
	AB24-1 to 6	light	Vyšenské kopce National Nature Reserve, South Bohemia – reared	MN107404 – MN107409	550	7151
A. agestis	JB00137	dark	Čepičná Nature Reserve, South Bohemia	MN107393	500	6747
	ZF-LY-001779	dark	Koněprusy, Čertovy schody quarry, Central Bohemia	MN107390	400	6050
	ZF-LY-001785	dark	Martinice, Velké Meziříčí, Vysočina region	MN107392	470	6662
	ArAg2	dark	Čimické údolí Nature Monument, Prague	MN107394	250	5852
	ArAg1	dark	Prokopské údolí Nature Reserve, Prague	MN107395	250	5952
	JB00124	light	Nerestský lom Nature Monument, South Bohemia	MN107396	470	6450
	ZF-LY-001784	light	Černousy, Frýdlant, Liberec region	MN107391	250	4956
	AB30-23	light	Nová Ves, Litovel, Olomouc region	MN107384	300	6368
	AB30-28	light	Mohelenská hadcová step National Nature Reserve, Vysočina region	MN107385	350	6863
	AB44-8	light	Hraniční hill, Město Albrechtice, Moravian-Silesian region	MN107386	500	5871
	AB44-9	light	Město Libavá, Olomouc region	MN107387	550	6271
	AB44-10	light	Chomýž, Krnov, Moravian-Silesian region	MN107388	350	5871
	AB44-11	light	Toužínské stráně Nature Monument, South Bohemia	MN107389	470	6958
	KA-2698	light	Havranické vřesoviště, Podyjí National Park, South Moravia	MN107397	330	7162

DNA was extracted from the legs using the Genomic DNA Mini Kit – Tissue (Geneaid) following the manufacturer's protocols. Using the Polymerase Chain Reaction (PCR), we amplified the mitochondrial gene cytochrome c oxidase subunit 1 gene (barcode/COI). We used the primer pair LCO/Nancy; or LCO/K699 and RON/HCO in case of fragmented DNA (primers: Monteiro and Pierce 2001; Wahlberg and Wheat 2008). We prepared the PCR mixture in 12.5 µl volume (6.25 µl Bioline 2× MyTaq HS Red Mix, 4 µl PCR H2O, 0.625 + 0.625 µl primers; 1 µl DNA). The thermal cycling profile was 95 °C for 5 min; then 40 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 90 s; and final extension 72 °C for 10 min. PCR products were cleaned with enzymes FastAP and ExoI (Thermofisher) and sequenced in one direction in Macrogen Inc. on ABI3730XL DNA analysers. Sequences were checked visually and aligned in GENEIOUS v. 8.0.5 (Kearse et al. 2012). Obtained sequences were submitted to the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/; accession codes MN107385–MN107409, Suppl. material 1).

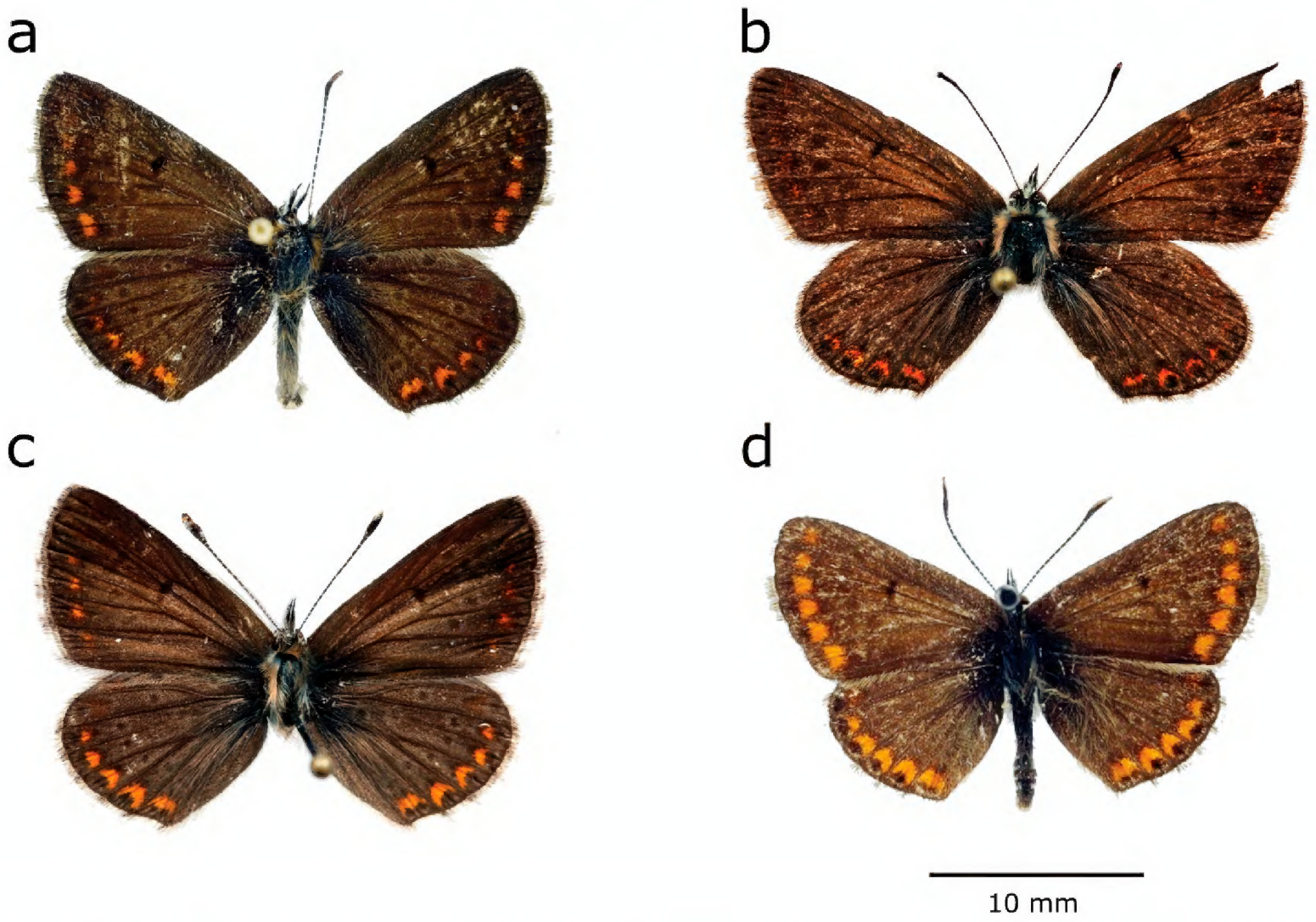

Figure 2. Examples of the sequenced specimens of *Aricia artaxerxes* and *A. agestis*. **a.** *Aricia artaxerxes*, (ZF-LY-001623, MN107399, Vyšenské kopce National Nature Reserve). **b, c.** *Aricia agestis*, darkly coloured individuals (**b.** JB00137, MN107393, Čepičná Nature Reserve. **c.** ZF-LY-001785, MN107392, Martinice, Velké Meziříčí). **d.** *Aricia agestis*, a typical, lightly coloured individual with developed orange spots (AB44-11, MN107389, Toužínské stráně Nature Monument).

The BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with GenBank reference database assigned the sequences to either *A. artaxerxes* or *A. agestis*. MEGA7 (Kumar et al. 2016) was used for computing p-distances (i.e., proportion of nucleotide site differences) among sequences and groups of sequences (species). We mined 94 sequences of *A. artaxerxes* from GenBank (Suppl. materials 1, 3), which we used, together with the twelve sequences of Czech *A. artaxerxes*, to construct the TCS statistical parsimony haplotype network (Clement et al. 2000) in the program POPART (Leigh and Bryant 2015). Using one sample of each *A. artaxerxes* haplotype from GenBank, together with nine randomly chosen samples of European *A. agestis* and representatives of *A. anteros*, *A. cramera* and *A. montensis* (Suppl. materials 1, 2), we reconstructed the Maximum Likelihood tree of all tested specimens using the IQTREE web server (Nguyen et al. 2015; Trifinopoulos et al. 2016) with ultrafast bootstrap (Minh et al. 2013).

Results

All darkly coloured as well as the reared light putative *Aricia artaxerxes* individuals from Vyšenské Kopce NNR reserve (N=12) were unequivocally *A. artaxerxes* according to both BLAST and Maximum Likelihood analysis (Fig. 3). All other tested individuals, both darkly- and lightly-coloured,

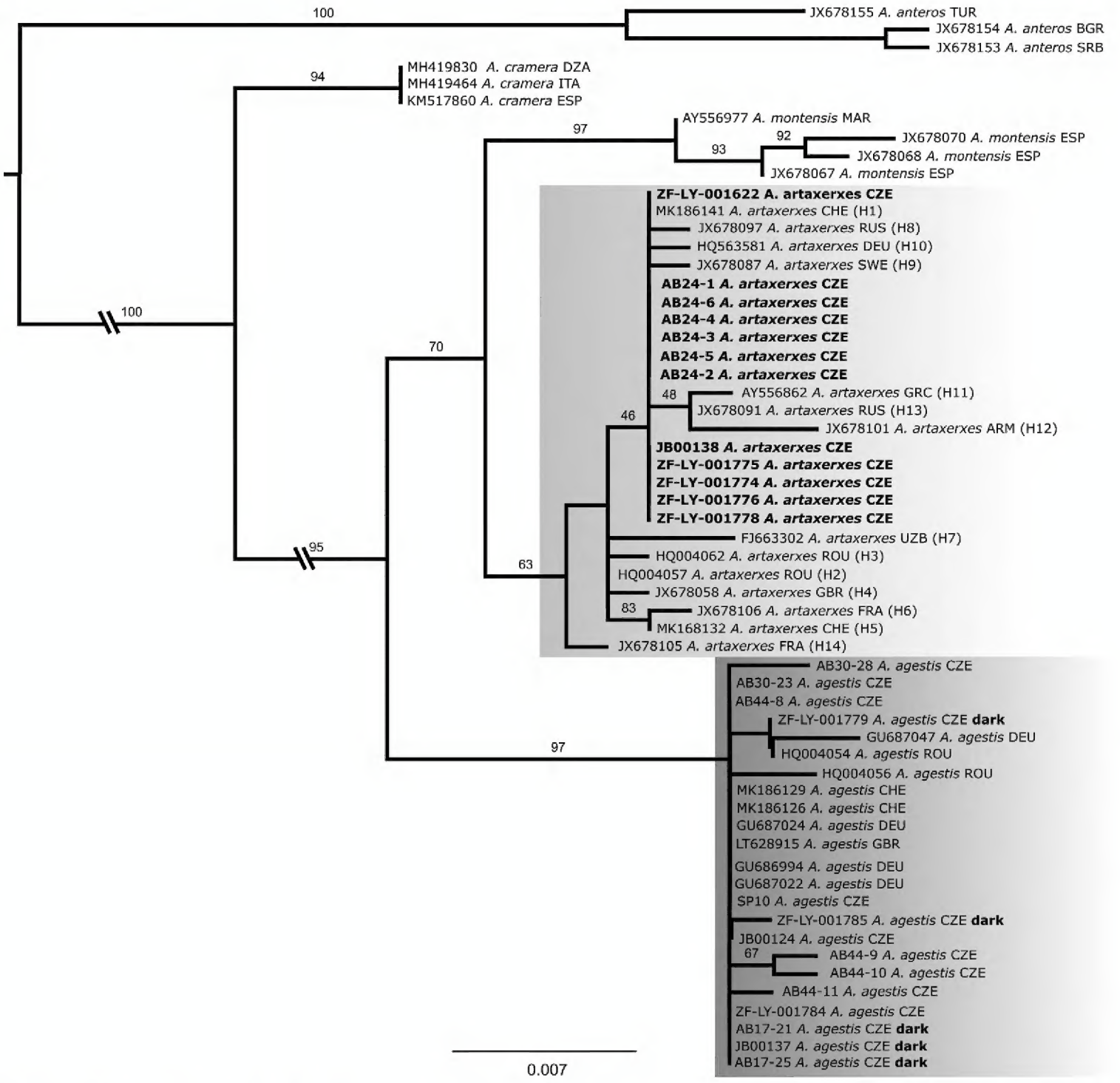

Figure 3. Maximum Likelihood tree of the Czech Republic *Aricia* spp. butterflies. The tree is based on cytochrome c oxidase subunit I (DNA barcode) sequences obtained for this study (Czech material) and retrieved from GenBank. The sample AY556977 from Morocco is labelled as *Aricia artaxerxes* in GenBank, This specimen was renamed as *A. montensis*, which occurs in North Africa (Sañudo-Restrepo et al. 2013). Branch labels show bootstrap values.

were *A. agestis*. The minimum p-distance between Czech *A. artaxerxes* and *A. agestis* was 1.98% and the mean p-distance was 2.10%.

The Czech *Aricia artaxerxes* population is monomorphic, consisting of a single COI haplotype (Fig. 4). Comparing the published barcodes of *A. artaxerxes*, this haplotype (H1) is widely distributed in the Palaearctic (Central Asia, Western Russia, Crimea, Central Germany, both Eastern and Western Alps and European North), and its derived haplotypes (Haplogroup A) are present in Greece, Baden-Württemberg, and reach the Far East. The other widespread haplotype (H2) and its derived haplotypes have a more southerly distribution – in the Balkans, Alps, and reach the United Kingdom. The highest haplotype diversity is in the Western Alps where five different haplotypes are present in twelve samples. However, as reported previously (Sañudo-Restrepo et al. 2013), the overall p-distance is low within this species (mean = 0.20%, max = 1.10%).

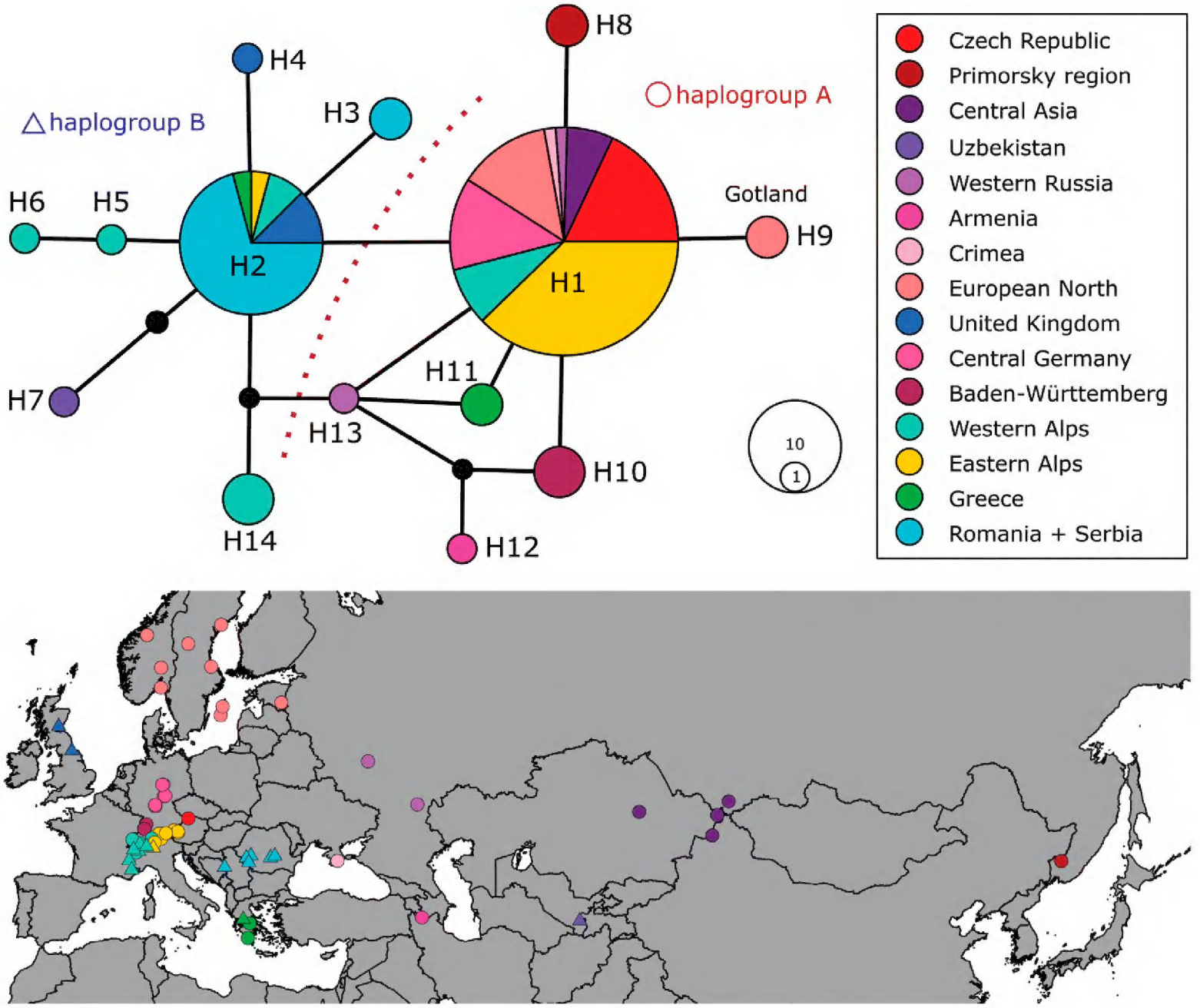

Figure 4. TCS haplotype network of *Aricia artaxerxes*. The network is based on cytochrome c oxidase subunit 1 (DNA barcode) sequences obtained for this study (Czech material) and retrieved from GenBank. Circles represent individual haplotypes, black dots represent putative haplotypes (not found among samples), and each connecting line represents one mutation.

Discussion

Using DNA barcoding, we confirmed that the putative *Aricia artaxerxes* population from Vyšenské Kopce NNR, Czech Republic, indeed belongs to this species, whereas other darkly-coloured samples from the country belong to *Aricia agestis* (Fig. 2). The fact that the reared individuals of *A. artaxerxes* resemble *A. agestis* suggests a phenotypic plasticity also in this species. Moreover, the Czech population is situated at a relatively low elevation (ca. 550 m A.S.L.). Relying on wing morphology or elevational records thus can lead to misidentifications between these two species. Our results highlight the need for DNA-based identification of these two species within their overlapping range (cf. Dincă et al. 2011).

Besides providing confirmation of *A. artaxerxes* distribution, our results indicate that its Czech population is isolated by ca. 190 km from the nearest genetically confirmed population of the species in Europe, possesses an extremely narrow distribution, and rightly deserves its critically endangered status (cf. Hejda et al. 2017).

This finding raises the national and even Central European importance of the Vyšenské Kopce NNR (area: 66.7 ha), plus two reserves within its closest environs (Výří vrch Nature Monument 12.9 ha, and Cvičák Nature Monument, 61.4 ha), for butterfly conservation. These three reserves, parts of the Blanský Les Landscape Protected Area, protect a system of (sub)xerophilous grasslands, shrublands and sparse woodlands on metamorphous limestones, a rare phenomenon within the prevailingly base-poor, cold and damp north-eastern foothills of the Šumava Mts. These calcareous localities have been rescued from successional overgrowth by restoration activities, partly depending on volunteers, since the 1970s onwards (Albrecht 2003); the Cvičák Nature Monument was for a time used by the military (cf. Čížek et al. 2013). The current conservation management of these sites is exemplary, aiming at retaining a dynamic mosaic of coppiced woodlands, scrub and grasslands maintained by mowing and goat and cattle grazing of varying intensity. The reserves host a regionally outstanding butterfly diversity (Hanč 2005, 2011; Slámová et al. 2013). Within this system, *A. artaxerxes* inhabits short-sward, open-turf and flower-rich sites with dense growth of *Helianthemum grandiflorum obscurum* (most likely the local larval host), which cover an area not exceeding 20 ha in total (Fig. 5a).

The entire area belongs to a wider system of calcareous islets adjoining the Šumava Mts. in an approximately NW-SE direction, and supporting multiple range-restricted relict xerophilous plants and animals (Fig. 5b). These include species with southern distribution (e.g., the bush-cricket *Leptophyes bosci* Fieber, 1853 (Holuša et al. 2013)); south-western elements believed to have colonised Czech territory via south-western mountain passes (e.g., the lycaenid butterfly *Pseudophilotes baton* (Bergsträsser, 1779) (Konvička et al. 2008)); and alpine floral elements that likely descended to the Danube lowlands in colder periods of the Pleistocene (e.g., *Verbascum chaixii* subs. *austriacum* (R. et Sch.) Hayek (cf. Holub and Skalický 1959; Kaplan 2012)). The population of *A. artaxerxes* appears as another example of this "perialpine" element, a relict of a time when the currently high-elevation species descended to northern foothills of the Alps (Schmitt 2017). Our *Aricia* sp. samples included material from yet another calcareous islet of the Šumava Mts. foothills (Čepičná, see Table 1). Although the presence of *A. artaxerxes* has not yet been confirmed there, the possibility of its occurrence there or in other localities makes further surveys desirable.

On the other hand, we are sceptical regarding the earlier (1970s–1980s) records of *A. artaxerxes* from eastern parts of the Czech Republic (south-eastern Moravia). The records were not followed by subsequent observations, despite intensive recording in the region (e.g., Bělín and Gottwald 2001; Konvička et al. 2008; Spitzer and Beneš 2010; Uřičář et al. 2016). We have shown here that even *A. agestis* occasionally forms darker variants (Fig. 2). In addition, no *A. artaxerxes* records from adjoining Western Slovakia are known to Slovakian colleagues; the closest known populations of putative *A. artaxerxes* are from Central Slovakia (near Východná and Važec), more than 100 km distant from the East Moravian localities (L. Víťaz, personal communication).

In the wider geographical context, the overall COI genetic diversity within *A. artaxerxes* is low when compared to its sibling *A. agestis* (Sañudo-Restrepo et al. 2013; Vodă et al. 2015).

The mitochondrial haplotype network (Fig. 4) does not show pronounced genetic structure within *A. artaxerxes* and the following interpretation needs to be taken with caution. The network suggests the possible existence of two haplogroups, differing by a single mutation only, distributed in wide longitudinal belts across the Palaearctic. The first (haplogroup A) is distributed from the Far East to Scandinavia, including also the Eastern Alps and the German and Czech populations. The second (haplogroup B) has a more southerly distribution, in Romania and the Western Alps, but also

Figure 5. Calcareous localities adjoining the Šumava Mts. chain. **a.** Habitat of the Czech Republic population of *Aricia artaxerxes*, short-sward calcareous grassland near Český Krumlov, South Bohemia. **b.** Calcareous localities adjoining the Šumava Mts. chain in the wider geographic context. Although numerous, all the localities represent small and relatively isolated patches within predominately acidic bedrock, and the majority of their summed area is forested. The closest contiguous limestone areas are the Northern Alps, situated to the south, and the Franconian Jura, situated to the west. Blue field and coloured dots: calcareous areas. Yellow dot: Čepičná Nature Reserve, Red dot: Vyšenské kopce National Nature Reserve.

in northern Great Britain. Both haplogroups are present in the Western Alps, where several unique haplotypes also exist, and in Greece. The presence of unique haplotypes suggests that the species survived the glacial maxima in these topographically diverse regions, possibly on mountain foothills (cf. Schmitt 2017). Moreover, these areas could also represent a secondary contact zone for the two haplogroups. Samples from the Italian Peninsula, which are still lacking in the dataset, could shed more light on this problem. The Palaearctic-wide distribution of haplogroup A could either point to a rapid postglacial expansion from a refugium, or to a more continuous distribution during glacials, as detected also for other continental species (Marešová et al. 2019; Bartoňová et al. 2018). We incline towards the second option, as postglacial dispersal from a restricted refugium to the wide belt stretching from the Alps to the Altai Mts. seems unlikely. Unique haplotypes were detected in still more southerly areas (H7 – Uzbekistan, H11– Greece, H12 – Armenia), but none was separated from the most frequent haplotypes (H1, H2) by more than three mutations. Their existence might indicate further structuring of populations in these areas, but more data are needed to ascertain this.

In any case, *Aricia artaxerxes* constitutes a boreomontane species with the European part of its range divided into a northern area where it frequents short-sward grasslands with the warmest microclimates, and a more southerly area where it inhabits localities of a similar character, but usually situated at high elevations. Adjoining the southern mountains, there exist isolated relict sites in relatively low elevations, potentially threatened by successional changes. This situation is remarkably similar to other butterfly species occurring both at high elevation and on piedmont grasslands, whose piedmont sites have been disappearing due to modern land use changes, whereas the high-elevation populations still hold on (e.g., *Lasiommata petropolitana* (Fabricius, 1787) (Spitzer et al. 2018) or *Parnassius apollo* (Linnaeus, 1758) (Todisco et al. 2010; Habel et al. 2012)).

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Supplementary material 1

The samples of *Aricia* spp. used for species identification in the Czech Republic and TCS haplotype network through its distributional range, including GenBank samples

Authors: Alena Sucháčková Bartoňová, Jiří Beneš, Zdeněk Faltýnek Fric, Martin Konvička

Data type: species data

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Link: https://doi.org/10.3897/nl.42.38853.suppl1

Supplementary material 2

NEXUS alignment of *Aricia* spp. cytochrome c oxidase subunit I sequences used for Maximum Likelihood analysis, covering samples from the Czech Republic and samples from GenBank

Authors: Alena Sucháčková Bartoňová, Jiří Beneš, Zdeněk Faltýnek Fric, Martin Konvička

Data type: molecular data

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Link: https://doi.org/10.3897/nl.42.38853.suppl2

Supplementary material 3

NEXUS alignment of *Aricia artaxerxes* cytochrome c oxidase subunit I sequences used for haplotype network analysis, covering samples from the Czech Republic and samples from GenBank

Authors: Alena Sucháčková Bartoňová, Jiří Beneš, Zdeněk Faltýnek Fric, Martin Konvička

Data type: molecular data

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